Handbook of Experimental Pharmacology 251

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Brown Adipose Tissue

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Brown Adipose Tissue

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Preface

Spotlight on Brown Adipose Tissue: Four Short Questions

What Is Brown Adipose Tissue?

This simple question is not so easy to answer, because brown fat is a special type of fat that has not much in common with the adipose tissue that we usually associate with "fat," i.e., the white adipose tissue. White fat is our largest store of energy, whereas brown fat generates heat – this process is also called non-shivering thermogenesis, because it does not rely on muscle contraction and shivering. Energy expenditure by brown fat is physiologically induced by cold stress but can also be induced pharmacologically by the stress hormone norepinephrine. Brown adipose tissue is special because it expresses the unique uncoupling protein 1 (UCP1) that uncouples mitochondrial respiration from ATP production, thus generating heat. The location and quantity of brown fat vary with age and sex; it can be found mainly between the shoulder blades, in the neck, deep within the chest around the great vessels, and around the kidney.

What Is Beige Fat?

Adipose tissue exhibits a substantial degree of plasticity and depots can change their phenotype/color. White fat – especially the subcutaneous depot – can take on the appearance of brown fat. This process can be observed after cold exposure and is also known as "browning." These cells share many characteristics with brown adipocytes and are therefore called brown-in-white (thus the abbreviation brite) or beige cells. Importantly, beige fat can contribute to whole body energy expenditure, albeit to a lesser extent than brown fat.

Why Is BAT so Interesting at Present or Why Do We Need a Compendium of Reviews on BAT Now?

Obesity has reached pandemic dimensions, and there is a lack of specific and efficient pharmacological treatment of overweight and obesity. Thus, there is high medical need for novel treatment strategies, and increasing energy expenditure has been suggested to be a potential strategy to fight obesity. But how can energy expenditure be achieved? Telling obese people to exercise is obviously not enough. The publications in 2007 and 2009 showing that adult humans possess metabolically active brown fat and that its activity correlates with leanness sparked off new interest in this special type of fat. Many labs shifted their attention to brown and beige fat, and since the last decade a wealth of new studies have been published on this topic.

This review is especially dedicated to those scientists newly intrigued by the metabolic power of brown adipose tissue.

How Is This Handbook Structured?

The 21 articles of the handbook are arranged into four parts:

- Part I focuses on the differences in the development of brown versus beige adipocytes and how brown adipocytes can be cultured in vitro. The focus of the articles on adipocyte models lies on human brown adipocytes. In addition to technical aspects of lineage tracing in vivo, aspects of brown adipocyte aging are covered in this chapter.
- Part II centers on molecular mechanisms of BAT function, especially on UCP1, and signaling mechanisms. The latter encompass papers on novel lipid signals that control BAT and the second messengers cAMP that plays a major role in BAT activation and its "smaller sister/brother" cGMP, which is getting more attention as an important enhancer of brown adipocyte differentiation. This chapter also deals with the expanding field of noncoding RNAs (microRNAs and long-noncoding RNAs) of BAT and beige fat. It is well established that white fat secretes a broad spectrum of hormones and endocrine factors (e.g., leptin and adiponectin), and recent studies suggest that BAT also has endocrine functions.
- The function of BAT in human adults is still not completely understood. A major hurdle is the lack of efficient and cheap diagnostic markers that do not expose subjects to radiation (X-ray and radioactive tracer). This is the topic of Part III.
- Finally, Part IV deals with BAT activation in humans by foods and drugs. The handbook closes with a detailed review of the potential of BAT as a pharmacological target.

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Brown Adipose Tissue Development and Metabolism

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Abstract

Brown adipose tissue is well known to be a thermoregulatory organ particularly important in small rodents and human infants, but it was only recently that its existence and significance to metabolic fitness in adult humans have been widely realized. The ability of active brown fat to expend high amounts of energy has raised interest in stimulating thermogenesis therapeutically to treat metabolic diseases related to obesity and type 2 diabetes. In parallel, there has been a surge of research aimed at understanding the biology of rodent and human brown fat development, its remarkable metabolic properties, and the phenomenon of white fat browning, in which white adipocytes can be converted into brown like adipocytes with similar thermogenic properties. Here, we review the current understanding of the developmental and metabolic pathways involved in forming thermogenic adipocytes, and highlight some of the many unknown functions of brown fat that make its study a rich and exciting area for future research.

Keywords

Adipogenesis · Beige adipocyte · Brite adipocyte · Brown adipose tissue · Development · Glucose and lipid metabolism · Lineage tracing · Progenitor cells · Thermogenesis · Ucp1

1 Introduction and Background

1.1 Overview

Brown adipocytes, which reside in specific depots called brown adipose tissues (BAT), produce heat in a process called non-shivering thermogenesis. Thermogenesis in BAT is stimulated mainly by the sympathetic nervous system in response to cold exposure, and it helps maintain body temperature (euthermia) in placental mammals. The acquisition of BAT in early mammalian evolution is considered one key evolutionary advantage that allowed for the successful expansion of mammals, and its functional importance in newborn humans and small rodents has long been appreciated. More recently, it has become apparent that adult humans also have functionally relevant BAT and possibly the additional capacity to induce the formation of brown-like adipocytes within white adipose tissues (WAT) (called brite or beige adipocytes) under certain conditions. Because these thermogenic cells, when active, have a high rate of nutrient consumption and energy expenditure, their existence in adult humans not only correlates with improved metabolic profiles (Betz and Enerback 2018) but has stimulated interest in targeting them therapeutically to fight obesity and improve glycemic control (Hanssen et al. 2015; Ouellet

et al. 2012; Yoneshiro et al. 2011b, c). This has gone hand-in-hand with renewed interest in understanding the basic biological mechanisms of brown fat development and metabolic regulation, which includes understanding the cellular lineages and precursor cell pools that give rise to brown and brite/beige adipocytes, and the signals that govern their fuel selection and unique metabolism. Identifying brown adipocyte stem and progenitor cells, and elucidating the mechanisms that stimulate their differentiation into mature thermogenic adipocytes, could have important implications in developing brown fat-based therapeutics. Here, we will discuss our present understanding of brown adipocyte development and function, the related topic of brite/beige adipocytes, and key future goals and unanswered questions especially as they relate to potential therapies.

1.2 Basics of Non-shivering Thermogenesis

Cold-stimulated non-shivering thermogenesis (NST) in the brown adipocyte is dependent upon the intrinsic expression and function of uncoupling protein 1 (UCP1), an inner mitochondrial membrane transporter that dissipates the energy stored in the mitochondrial electrochemical gradient as heat, "uncoupled" from ATP synthesis (Betz and Enerback 2018). In the absence of thermal stress, brown adipocyte UCP1 is thought to be inhibited by purine nucleotides (Nicholls 2006; Sluse et al. 2006). During cold stress, brown fat thermogenesis is classically stimulated by norepinephrine released from the sympathetic nervous system (SNS), which activates β3-adrenergic receptors on brown adipocytes to stimulate intracellular synthesis of the second messenger cyclic AMP (cAMP), leading to cAMP-driven protein kinase A (PKA) signaling activation. This stimulates lipid catabolism processes such as lipolysis which liberates free fatty acids from triacylglycerol lipid storage droplets and increases expression of a thermogenic gene expression program that includes UCP1 mRNA (Nicholls 2006; Sluse et al. 2006; Fedorenko et al. 2012; Lehr et al. 2006).

Exactly how brown adipocytes choose and utilize fuel remains an important and open question. Recent studies suggest that active lipolysis in brown adipocytes may not be required for sustaining thermogenesis so long as exogenous lipids are available; nevertheless, cellular free fatty acids reportedly directly activate UCP1 (Fedorenko et al. 2012; Shin et al. 2017; Schreiber et al. 2017). Active brown adipocytes also take up glucose from circulation and synthesize free fatty acids de novo from glucose and possibly other lipogenic precursors (i.e., the process of de novo lipogenesis) to continuously fuel NST or to provide other yet-to-be-appreciated metabolic advantages (Sanchez-Gurmaches et al. 2018; McCormack and Denton 1977; Mottillo et al. 2014; Shimazu and Takahashi 1980; Trayhurn 1979; Yu et al. 2002). In addition, BAT thermogenesis is fueled by liver-derived plasma-lipid metabolites (acyl-carnitines), the release of which is stimulated by cold-induced lipolysis in the WAT (Simcox et al. 2017). It has also been suggested recently that UCP1-independent mechanisms of thermogenesis might exist under certain circumstances (Bertholet et al. 2017; Ikeda et al. 2018; Kazak et al. 2015). Brown adipocytes might also have key metabolic functions in addition to

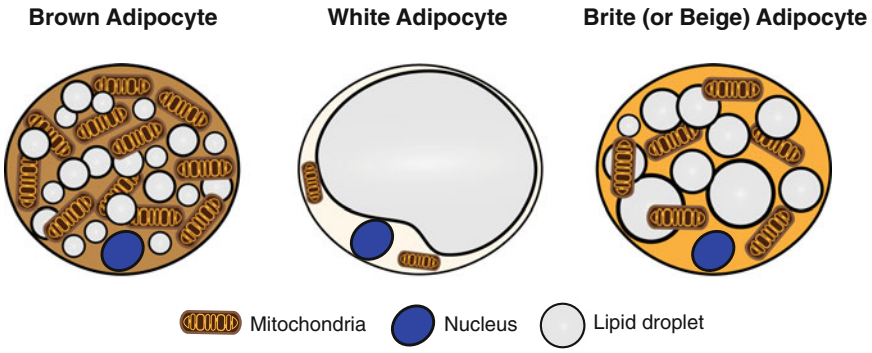
thermogenesis, such as secreting special adipokines (called BATokines) and exosomes containing miRNAs that might have both autocrine function and paracrine functions on nearby immune cells, as well as endocrine functions related to glucose homeostasis and cardiovascular health (Thomou et al. 2017; Villarroya et al. 2013; Hansen et al. 2014; Svensson et al. 2016; Long et al. 2016; Wang et al. 2014a; Villarroya and Giralt 2015).

1.3 Brown Fat Anatomy and Morphology

The color distinction between a "brown" and a "white" adipocyte largely reflects the many more mitochondria (which are high in iron) in brown adipocytes compared to white adipocytes (Fig. 1). A stimulated brown adipocyte actively generating heat also contains many small lipid droplets and is referred to as being multilocular, while white adipocytes, such as those in subcutaneous and visceral depots (sWAT and vWAT, respectively), typically have a single large unilocular lipid droplet (Fig. 1). Having many small lipid droplets increases lipid droplet surface area and presumably promotes metabolite exchange with mitochondria (Blanchette-Mackie and Scow 1983; Benador et al. 2018). A less active brown adipocyte that is not engaged in thermogenesis (e.g., after acclimation to thermoneutrality) adopts a morphology more similar to a white adipocyte although it retains an epigenetic cellular identity that differentiates it from a white adipocyte (Roh et al. 2018).

As indicated above, brown adipocytes exist in defined BAT depots in the mouse, which is the main model organism used to study brown fat. Notably, the size and composition of each BAT depot differs with age, gender, and mouse strain background (Frontini and Cinti 2010; Murano et al. 2009). The largest BAT depots are clustered in the dorsal anterior regions of the mouse body and include the interscapular (iBAT), subscapular (sBAT), and cervical depots (cBAT) (Frontini and Cinti 2010; de Jong et al. 2015; Walden et al. 2012; Cinti 2005) (Fig. 2). In addition, there are several small BAT depots proximal to major blood vessels and specific organs, such as the periaortic BAT depot (paBAT) that aligns to aortic vessels and the perirenal BAT depot (prBAT) that localizes in a fibrous capsule of the kidney (Frontini and Cinti 2010) (Fig. 2). Recent studies using ^{18}F-FDG PET-CT or ($^{123/125}$I)-β-methyl-p-iodophenyl-pentadecanoic acid with SPECT/CT imaging, which traces glucose and lipid uptake, respectively, suggest additional small pockets of cold responsive fat depots exist in suprascapular, supraspinal, infrascapular, and ventral spinal regions (Zhang et al. 2018; Mo et al. 2017).

Similar to the mouse, newborn humans have active brown adipocytes present at birth in large interscapular BAT depots and perirenal depots (Fig. 3a), which presumably help maintain core body temperature though could also have other neonatal functions not yet appreciated. Until recently, it was widely believed that after neonatal BAT recedes, adult humans lacked brown fat. However, about a decade ago, the widespread existence of active BAT in adults was revealed by retrospective analyses of ^{18}F-fluodeoxyglucose (FDG) uptake assays, which use positron emission tomography-computed tomography (PET-CT) to measure glucose uptake into organs (Yoneshiro et al. 2011a, 2013; van der Lans et al. 2013; Ouellet



	Brown	*White*	*Brite/beige*
UCP1 Expression	Positive	Negative	Positive
Mitochondrial Density	High	Low	Medium
LD Morphology	Multi-locular	Uni-locular	Multi-locular
Primary Function	Thermogenesis Endocrine	Energy storage Endocrine	Thermogenesis? Endocrine?

Fig. 1 General characteristics of brown, white, and brite/beige adipocytes. A stimulated brown adipocyte (left) contains numerous small lipid droplets and many mitochondria and expresses high levels of uncoupling protein 1 (UCP1), which is embedded in the inner mitochondrial membrane and required for thermogenesis. The color of brown fat reflects the high iron content of mitochondria. A white adipocyte (middle) in contrast contains a single large lipid droplet and fewer mitochondria, and does not express UCP1. A brite/beige adipocyte (right) is characteristically intermediate between brown and white adipocyte, having multiple lipid droplets (though often larger than those seen in a brown adipocyte), more mitochondria than a white adipocyte, and it expresses UCP1

et al. 2012; Hanssen et al. 2015; Nedergaard et al. 2007; Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Saito et al. 2009; Virtanen et al. 2009; Kortelainen et al. 1993). These studies also revealed a correlation between BAT activity/amount and metabolic fitness. More recent studies show that BAT depots in adult humans exist in the supraclavicular, axillar, and paravertebral regions, though the variability across individuals and populations is still being worked out (Zhang et al. 2018; Nedergaard et al. 2007; Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009; Ouellet et al. 2012) (Fig. 3b). There are also small BAT depots in perivascular regions (aorta, common carotid artery), and near the heart wall (epicardium), lung bronchia, and some solid organs (hilum of kidney and spleen, adrenal, pancreas, liver) (Sacks and Symonds 2013) (Fig. 3b).

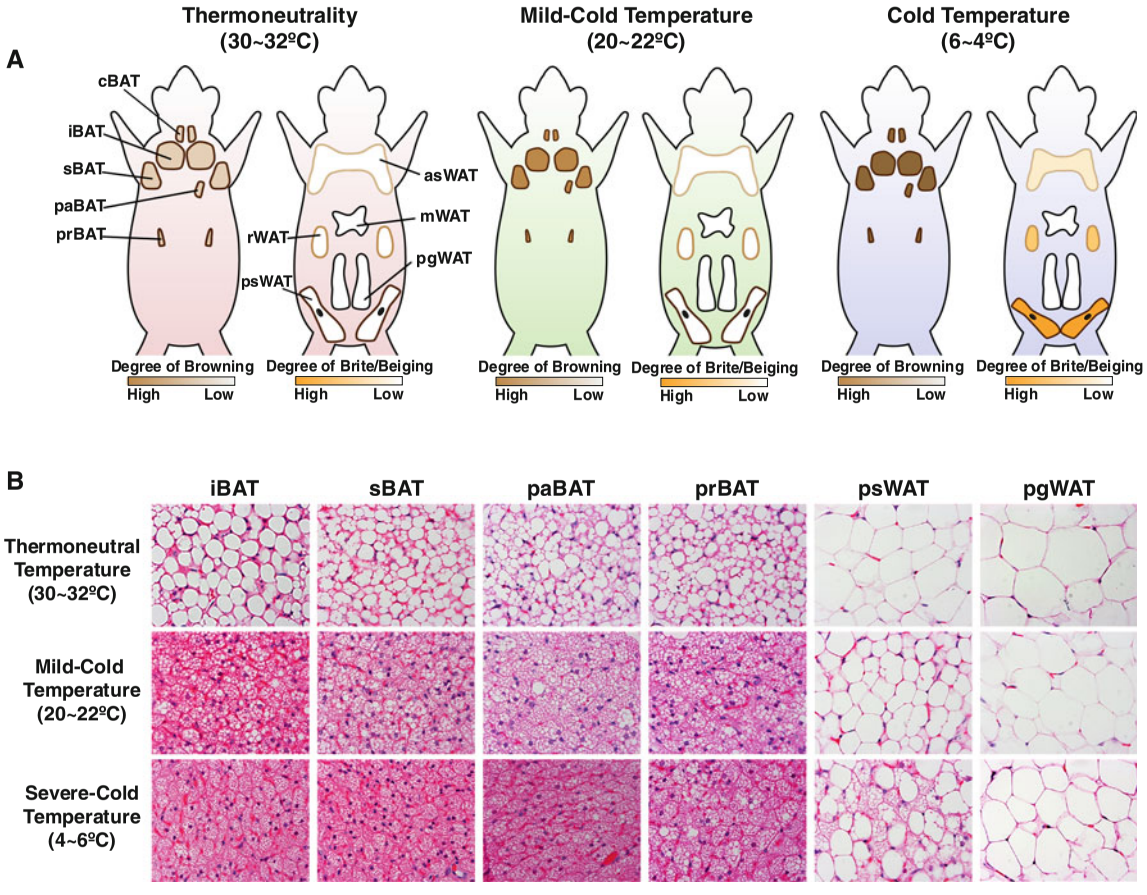


Fig. 2 Adipose tissue anatomy and plasticity. (**a**) Cartoons showing brown and white fat depots in mice that are acclimated to thermoneutrality (30–32°C), mild cold (20–22°C), and severe cold (4–6°C). The color and size of each depot is modeled such that it reflects the observed differences in mice acclimated to each

Fig. 2 (continued) temperature. A key showing the gradient of "browning" or "britening/beiging" is provided below each model. (**b**) Hematoxylin and eosin staining of the indicated brown and white fat depots at each temperature. Note that at thermoneutrality, brown adipocytes contain larger single lipid droplets. At 20–22°C, the standard mouse facility temperature, brown adipocytes exhibit their stimulated morphology of being multilocular (see Fig. 1), while white adipocytes remain unilocular though SWAT adipocyte size is reduced likely reflecting in part a higher level of lipolysis that is necessary to fuel the active brown fat depots. At severe cold temperatures (6–10°C), additional morphological changes can been seen in BAT (i.e., lipid droplets become more uniform), and under these conditions, brite/beige adipocytes also form in the subcutaneous WAT. Of note, the browning capacity of WAT depots is not dependent on a depot being subcutaneous or visceral because, for example, the retroperitoneal visceral WAT depot has high britening/beiging capacity (not shown) while the perigonadal visceral WAT (shown) does not. *iBAT* interscapular BAT, *sBAT* subscapular BAT, *cBAT* cervical BAT, *paBAT* periaortic BAT, *prBAT* perirenal BAT, *asWAT* anterior subcutaneous WAT, *psWAT* posterior subcutaneous WAT, *mWAT* mesenteric WAT, *rWAT* retroperitoneal WAT, *pgWAT* perigonadal WAT. The images in this figure are based primarily on experiments with C57Bl/6 mice

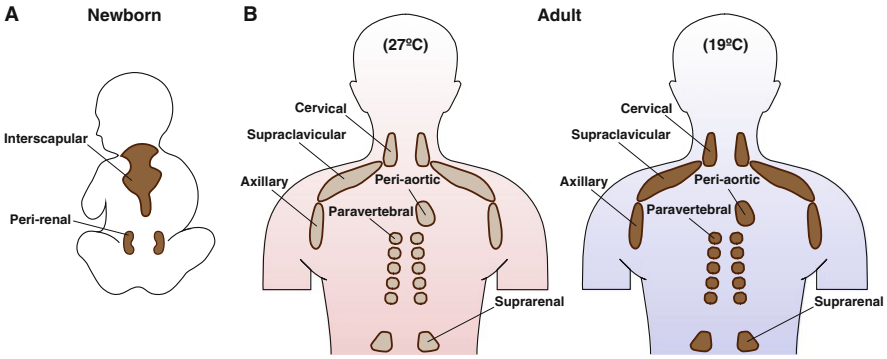


Fig. 3 Brown fat locations in humans. (**a**) Newborn infants have large interscapular and perirenal BAT depots. (**b**) In adults, smaller BAT depots are located in the cervical, supraclavicular, axillary, periaortic, paravertebral, and suprarenal regions. The mapping of these depots in adults is largely based on glucose uptake measurements by ^{18}F-FDG-PET-CT imaging, which shows increased glucose flux at colder temperatures (shown in figure) and on postmortem resections. The molecular and functional nature of individual (putative) BAT depots remains unclear in humans. Also note that the amount of BAT is highly variable between individuals, but when active BAT is present, it has been shown to correlate with improved metabolism (not shown, discussed in text). Emerging advances in BAT imaging will likely confirm additional depots

1.4 BAT Vascularization and Innervation

Brown fat depots are also highly vascularized, which facilitates the exchange of oxygen and nutrients and the dissipation of heat and release of BATokines into circulation (Bartelt et al. 2011; Labbe et al. 2015; Sacks and Symonds 2013). In fact, BAT requires increased blood infusion rate during BAT recruitment (i.e., cold stimulation) to obtain sufficient metabolic substrates and oxygen. Brown adipocytes also generate vascular endothelial growth factor-A (VEGF-A) and nitric oxide (NO), which facilitates BAT angiogenesis and vascularization (Xue et al. 2009; Sun et al. 2014; Nisoli et al. 1998; Mahdaviani et al. 2016), a process that is reduced in obese mice resulting in loss of thermogenic activity (Shimizu et al. 2014). Other recent work suggests that brown adipocytes may have a vasoprotective role that might be mediated by the secretion of hydrogen peroxide (H_2O_2), which inhibits vessel contractions in nearby vascular cells (Friederich-Persson et al. 2017).

In addition to being highly vascularized, BAT is extensively innervated allowing for its rapid stimulation by the sympathetic nervous system (SNS). The SNS releases catecholamines such as norepinephrine that activate G-protein-coupled β3-adrenergic receptors that are highly expressed on mature brown adipocytes and β1-adrenergic receptors on brown adipocyte precursors (Cannon and Nedergaard 2004; Morrison et al. 2012; Bukowiecki et al. 1986; Bronnikov et al. 1992). While β3-adrenergic receptor signaling stimulates mature brown adipocyte lipid catabolic activity and thermogenesis, β1-adrenergic receptor signaling stimulates brown fat adipogenesis upon prolonged cold challenge (Bronnikov et al. 1992). Classic denervation studies reveal the indispensability of the SNS connections for thermogenesis (Silva and Larsen 1983; Rothwell and Stock 1984; Takahashi et al. 1992; Labbe

et al. 2015). Emerging research also suggests that innervation may also be critical for BAT to communicate directly with other non-SNS tissues, such as the WAT (Schulz et al. 2013; Garretson et al. 2016; Nguyen et al. 2018).

In summary, BAT is a dynamic and heterogeneous tissue, and the extensive networks of vessels and nerves found in BAT suggest that during brown fat development, there is tight coordination between brown adipocyte precursors (discussed below), endothelial lineages, and nerve cell lineages and likely immune cells too (Lumeng and Saltiel 2011; Olefsky and Glass 2010; Villarroya et al. 2018). The signaling and metabolic interactions between different cell lineages during brown fat development have not yet been extensively studied by system-based approaches.

1.5 Transcriptional Control of Brown Adipocyte Differentiation

Much of the general transcriptional cascade that promotes adipogenesis is shared between brown and white adipocytes and has been studied at length using in vitro models (e.g., 3T3-L1 cells). The master regulator of adipogenesis, PPARγ, is both necessary and sufficient for adipogenesis (Rosen et al. 1999; Tontonoz et al. 1994; Wang et al. 2013a). Other key components of the general adipogenesis transcriptional cascade also important in brown and brite/beige adipocyte differentiation include the members of the C/EBP family (C/EBPα, C/EBPβ, C/EBPδ) (Farmer 2006). While PPARγ is the dominant factor, overexpression of all C/EBP family members induces adipocyte formation. In culture, C/EBPβ and C/EBPδ function in the first wave of adipogenic transcription factors (hours after adipogenic induction) that eventually triggers a second wave (days after adipogenic induction) that includes C/EBPα and PPARγ, which feed-forward activate themselves (Farmer 2006).

More recently, efforts to identify brown adipocyte lineage-specific transcription factors have identified new additional components that may contribute to the brown (or brite/beige) adipocyte fate. PRDM16 (PRD1-BF1-RIZ1 homologous domain containing 16) was originally described as a BAT transcriptional determination factor that induces a robust thermogenic adipocyte phenotype in white adipocytes both in vitro and in vivo and can direct muscle precursors to differentiate into brown adipocytes in vitro (Seale et al. 2007, 2008, 2011). In vivo, other PRDM family members can compensate for the loss of PRDM16 in BAT precursors to maintain normal BAT formation (Harms et al. 2014). In addition, the EBF2 (early B-cell factor 2) transcription factor is selectively expressed in both BAT and brite/beige precursors, and it is required for BAT identity and efficient brite/beige cell formation (Rajakumari et al. 2013; Stine et al. 2016; Wang et al. 2014b). Recent studies also identified zinc-finger protein 516 (Zfp516), whose expression in brown fat is markedly increased in response to cold exposure or β-adrenergic stimulation via β-AR-cAMP pathway, and it directly interacts with PRDM16 to promote BAT development and WAT browning while suppressing myogenesis (Dempersmier et al. 2015; Sambeat et al. 2016). Whether there are brite-/beige-specific

transcription factors that do not function in brown adipocyte lineages remains an important area of investigation.

In contrast to pro-thermogenic transcription factors, less is known about the transcriptional machinery that promotes and/or maintains the white adipocyte phenotype. One interesting candidate is Zfp423, which has recently emerged as a critical brake that prevents white adipocytes from converting to thermogenic adipocytes. Zfp423 is expressed in white adipocyte precursor cells and functions to block the brite/beige thermogenic program by inhibiting the EBF2 and PRDM16 (Gupta et al. 2010, 2012; Shao and Gupta 2018; Shao et al. 2016). While these studies are opening the door to our understanding of adipocyte fate determination at the level of gene expression, there is still much to be learned especially if this information is to be harnessed for therapeutic opportunities. Moreover, other key gene expression factors that contribute to fate decisions, such as epigenetic marks and higher-order chromatin regulation, are just beginning to be explored (Roh et al. 2017, 2018; Zhao et al. 2016; Carrer et al. 2017) making this an important area of investigation for many years to come.

2 Brown Fat Growth

2.1 Techniques for Studying BAT Development

Understanding how brown fat grows begins with understanding its developmental origins. We begin this section with a brief commentary on the two main methods that have been instrumental in beginning to elucidate the developmental origins of both brown and white adipocytes: (1) *fluorescence-activated cell sorting (FACS)*, in which stem and progenitor cells are isolated based on their expression of cell surface markers or engineered genetic labels and then tested for their ability to function as adipocyte precursors, and (2) *lineage tracing*, in which stem and progenitor cells are indelibly labeled with a genetic mark that can be followed, or traced, throughout development in all descendant cells.

2.1.1 FACS

Adipocyte precursors reside within whole adipose tissue depots in a highly heterogeneous non-adipocyte cell population commonly referred to as the stromal vascular fraction or "SVF." In addition to adipocyte stem and progenitor cells, the SVF contains endothelial, immune, nerve, and other cells that support tissue function. Adipocyte precursors are necessary not only for establishing fat depots but also for expanding and regenerating adipocytes. Starting with only the SVF population from white adipocytes, several studies have used FACS technology with cell surface markers thought to label the adipocyte precursor population to enrich for pools of adipocyte stem and progenitor cells (ASPCs) (Berry et al. 2014). Although a single marker for prospective isolation of adipocyte precursors has not been found, combinations of surface markers have been use in this regard to isolate white ASPCs (Berry and Rodeheffer 2013; Rodeheffer et al. 2008). One common

example in mice is the CD31^{neg}, CD45^{neg}, Ter119^{neg}, CD29^{pos}, Sca1^{pos}, CD34^{pos}, and CD24^{pos} population, which has enhanced adipogenic potential compared to the total SVF. Although brown and white adipocytes have many functional, anatomical, and morphological differences, a similar population of ASPCs can be isolated from BAT depots (Sanchez-Gurmaches et al. 2012; Wang et al. 2014b).

Recently, PDGFRα was also reported to be a marker for ASPCs. PDGFRα can be used to isolate ASPCs using flow cytometry from the CD31^{neg} and CD45^{neg} population within the SVF of all WAT and BAT (Church et al. 2014; Berry and Rodeheffer 2013). These findings have been further validated using lineage-tracing approaches (discussed below), which confirm that adipocyte lineages express Cre recombinase driven by the PDGFRα promoter (Berry and Rodeheffer 2013; Vishvanath et al. 2016; Lee et al. 2012, 2015). From a technical perspective, this finding is important because it simplifies the enrichment protocol for ASPCs. Interestingly, PDGFRα also labels a fibro/adipogenic precursor cell population within skeletal muscles and skin (Joe et al. 2010; Rivera-Gonzalez et al. 2016) suggesting PDGFRα may be a broadly relevant marker of ASPCs, and recent studies further conclude that PDGFRα signaling may functionally contribute to ASPCs fate and adipose tissue organogenesis (Rivera-Gonzalez et al. 2016; Sun et al. 2017). However, PDGFRα also expresses in many non-adipocyte cells, and it will be important to delineate its different roles within the heterogeneous SVF population of adipose tissues.

A current key challenge of using FACS-isolated adipocyte precursors is that the ASPCs, although enriched for adipogenic precursors, are still a heterogeneous population containing subpopulations of cells that remain largely undefined by molecular approaches, and whether a true adipocyte stem cell can be purified is still an open question. Recent studies using single-cell RNA-seq are beginning to provide key insights into this problem (discussed below). Other studies have identified markers of differentiated brown or beige adipocytes (Ussar et al. 2014). However, highly specific and reliable surface markers that can differentiate between brown, beige, or white adipocyte progenitors have not yet been identified. On the other hand, the prospective nature of using FACS to isolated adipocyte precursors may facilitate the isolation and application of human ASPCs for use in cell-based therapies. Several different protocols for the isolation of human adipocyte progenitors are being developed (van Harmelen et al. 2005; Baglioni et al. 2009, 2012; Perrini et al. 2013).

2.1.2 Lineage Tracing

Lineage tracing is a classic developmental biology technique that has been used to study adipose tissue development mainly in mouse models due to its genetic nature. In classic lineage-tracing experiments, an indelible mark, often a fluorescent reporter, is expressed in a specific population of precursor cells by homologous recombination or by a transgenic approach, and the permanently modified cells then transmit the reporter to all of their descendant cells or lineages. A common strategy for studying adipocyte lineages in mice is to use a cell-specific Cre recombinase that activates the reporter's expression. Cre drivers can be always on in the specific

population being studied, called constitutive, or stimulated to be active only transiently in a cell population, called inducible. The latter requires the administration of a stimulus to turn on Cre activity.

Both methods have advantages and disadvantages that need to be considered for data interpretation of adipocyte lineages. For starters, there is currently no known Cre driver that only expresses in ASPCs. Inducible Cre recombinases have the advantage that they allow the timing of activation to be regulated such that cells are only labeled for a brief moment, and then those specific cells can be followed. This is not achievable with constitutive Cre drivers, making it difficult to determine precisely which cells first express the Cre in a particular lineage using these drivers. However, the inducers used to turn on the inducible Cre drivers, typically tamoxifen or doxycycline, can have unintended toxic effects on cells (Ye et al. 2015; Moullan et al. 2015). Even when pools of cells are inducibly labeled, it is difficult to distinguish whether two descendant labeled cells originate from common or distinct Cre-expressing precursors. Another consideration is whether a particular Cre driver reflects the expression of the actual endogenous gene/protein whose promoter is used to drive the Cre or whether it only reflects the promoter activity uncoupled from the normal expression of the associated gene and/or protein. The use of knock-in Cre drivers, which are expressed from endogenous promoters, can help mitigate against this concern. Related to this point, caution should be taken in inferring whether the activity of a specific Cre (i.e., promoter) reflects a functional role for the associated gene in lineage specification.

The choice of a reporter (often a fluorescent reporter) is also important when performing lineage tracing in adipocytes. One issue with adipocytes relative to non-adipocytes is the small amount of cytoplasm and large quantity of lipid droplets, which both make the use of cytoplasmic fluorescent reporters challenging to detect and make it difficult to obtain high-quality frozen sections. Thus, the reporter of choice for adipocytes is typically a membrane-targeted reporter, such as the dual-fluorescent membrane-targeted Tomato, membrane-targeted GFP, or mTmG reporter (Muzumdar et al. 2007). This reporter has two major advantages: (1) all cells are labeled, the mGFP reporter only being activated in Cre-positive lineages, and (2) both fluorescent reporters are membrane targeted. Its utility in adipose tissue both for lineage tracing as well as for use in FACS-based studies has been demonstrated in many reports (Berry and Rodeheffer 2013; Sanchez-Gurmaches and Guertin 2014; Shao et al. 2016; Wang et al. 2014b). Related to lineage tracing is cell-labeling or cell-marking, which is a common technique to study mature adipocyte dynamics. By this strategy, only mature adipocytes are labeled (rather than precursors), which allows single mature adipocytes to be followed over time especially when combined with inducible Cre drivers of reporter expression.

2.2 Brown Adipocyte Origins

Brown adipocytes are thought to originate from the mesoderm during embryonic development and thus share a very early developmental origin with skeletal muscle,

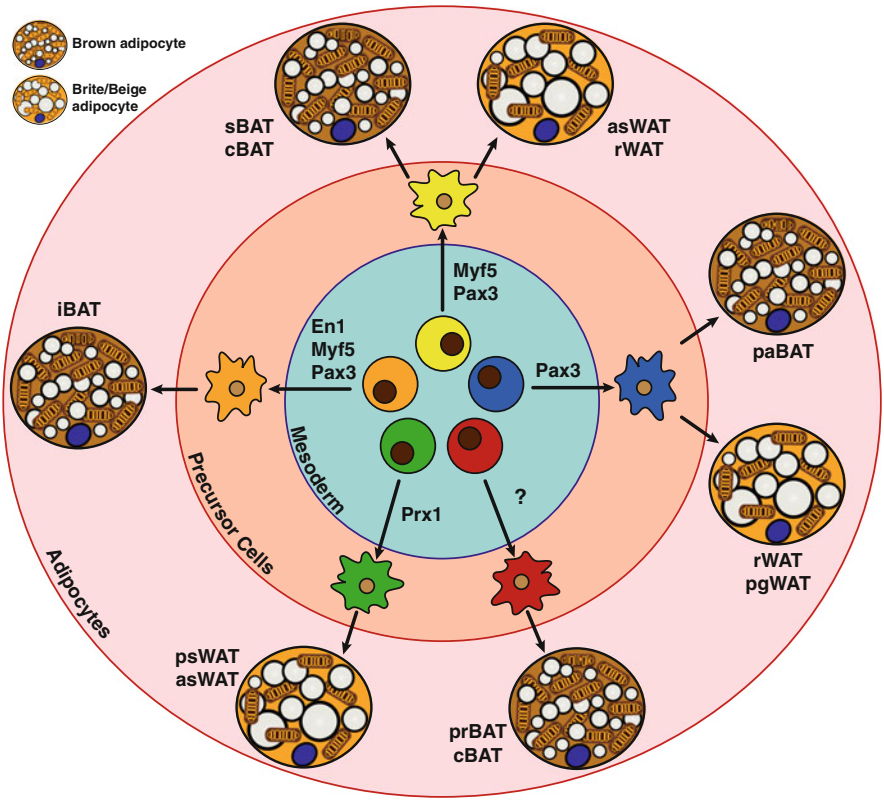


Fig. 4 Model of the heterogeneity and complexity in brown and brite/beige adipocyte development. Several multipotent cell populations that are mainly mesodermal and express specific transcription factors (e.g., En1, Myf5, Pax3, Prx1) appear to give rise heterogeneously to thermogenic adipocytes in different depots. Note that there is overlap shared with some markers but not with others. For example, Pax3 and Myf5 together may mark a pool of early precursors that give rise to iBAT, but only Pax3 marks a precursor pool that gives rise to some visceral pgWAT adipocytes (discussed in text). The significance of this heterogeneity is not understood. Additionally, there are several populations of brown and brite/beige adipocytes for which potential lineage markers remain unidentified. Also note that the brown and brite/beige adipocytes shown in this figure are depicted in their active state (i.e., upon β-adrenergic stimulation), but in vivo brown and brite/beige adipocytes are not necessarily present at the same time, such as in mild cold conditions (see Fig. 2)

bone, white adipocytes, and connective tissues (Wang et al. 2014b; Atit et al. 2006; Seale et al. 2008; Lepper and Fan 2010; Sanchez-Gurmaches et al. 2012). However, the pathways that specify the brown adipocyte developmental lineage is not fully clear. In accordance with a mesodermal origin, a population of cells within the central dermomyotome that is labeled at E9.5 by expression of the homeobox transcription factor Engrailed 1 (En1) gives rise to iBAT, dermis, and epaxial muscles (Figs. 2a, 4 and Table 1) (Atit et al. 2006). However, these E9.5 $En1^{+}$ progenitors do not appear to give rise to sBAT or any of the major white fat depots

Table 1 Depot-specific developmental origins of BAT

Tissue type	Anatomical locations (Human)	Anatomical locations (Mouse)	Developmental origins (Lineage tracing study)
Brown adipocyte	Supraclavicular/ paravertebral Perivascular: (aorta, artery) Periviscus: (heart, lung bronchia) Solid organs: Kidney/spleen hilum, pancreas, liver	Interscapular	$En1^{+}$, $Myf5^{+}$, $Pax7^{+}$, $Pax3^{+}$, $Prx1^{-}$
		Subscapular	$En1^{-}$, $Myf5^{+}$, $Pax7^{+}$, $Pax3^{+}$, $Prx1^{-}$
		Cervical	$En1^{?}$, $Myf5^{\pm}$, $Pax7^{+}$, $Pax3^{\pm}$, $Prx1^{-}$
		Perirenal	$En1^{?}$, $Myf5^{-}$, $Pax7^{+}$, $Pax3^{\pm}$, $Prx1^{-}$
		Periaortic	$En1^{?}$, $Myf5^{-}$, $Pax7^{+}$, $Pax3^{-}$, $Prx1^{\pm}$
Brite/beige adipocyte	Supraclavicular? Subcutaneous	Posterior subcutaneous (Inguinal)	$En1^{?}$, $Myf5^{-}$, $Pax3^{-}$, $Prx1^{+}$

(Atit et al. 2006) (Atit personal communication) suggesting that some brown and white adipocyte origins may differ and that not all brown adipocytes share a common origin (see below). This concept of adipocyte heterogeneity within and between depots, as we will discuss, is now a central tenet of adipocyte biology.

The model tilted toward brown fat and skeletal muscle sharing a common developmental origin with the finding that brown adipocytes in the iBAT and the skeletal muscles, but not certain populations of WAT, share a common cellular origin in the dermomyotome defined by the expression of Myf5-Cre (Seale et al. 2008). Using the constitutively expressing Myf5-Cre knock-in driver with a cytoplasmic reporter, this study found that Cre recombinase activity labels mature brown adipocytes in the iBAT in addition to skeletal muscles (Seale et al. 2008). Myf5 is a classic myogenic determination factor from the basic helix loop helix (bHLH) family, and thus the labeling of brown adipocytes with Myf5-Cre (Tallquist et al. 2000) was predicated to explain the metabolic similarities between brown fat and skeletal muscle with respect to high oxygen consumption and fuel usage and conversely the metabolic difference between BAT and the less metabolically active and energy-storing WAT depots (Harms and Seale 2013). Notably, at the time, most studies used mice that were mildly cold stressed in which the BAT is hyperactive, rather than mice living at thermoneutrality, when brown adipocytes are more similar morphologically and metabolically to white adipocytes. Nevertheless, in support of this model, an inducible Cre driver under control of the Pax7 promoter (the PAX transcription factor family member 7 collaborates with Myf5 and other myogenic factors during skeletal myogenesis) showed that Pax7+ progenitors that arise between E9.5 and E10.5 (but not later in development) also give rise to interscapular brown adipocytes (Lepper and Fan 2010). This also suggested an early divergence between BAT and muscle lineages.

While the Myf5-lineage model of BAT specification was elegant in its simplicity, studies challenging its uniformity soon after revealed that the brown adipocyte developmental landscape is more complicated. Similar fate-mapping experiments

using the same Myf5-Cre driver, but more broadly examining brown and white fat depots, and using the mTmG reporter, showed that many white adipocytes are also Myf5-Cre lineage positive and unexpectedly that many brown adipocytes are Myf5-Cre lineage negative (Sanchez-Gurmaches and Guertin 2014). For example, Myf5-Cre labeled precursors appear to give rise to nearly all brown adipocytes in iBAT and sBAT depots, but only about half of the brown adipocytes in the cervical BAT, and none of the brown adipocytes in prBAT or paBAT. Moreover, Myf5-Cre-positive adipocytes populate the asWAT and rWAT depots (Fig. 2a), indicating that Myf5-Cre neither uniformly nor specifically labels brown adipocytes. Other studies have replicated these findings confirming the heterogeneous labeling of adipocytes with Myf5-Cre (Sanchez-Gurmaches and Guertin 2014; Sanchez-Gurmaches et al. 2012; Shan et al. 2013; Wang et al. 2014b).

Interestingly, lineage tracing using a Pax3-Cre driver, (Pax3 is another myogenic Pax family transcription factor that expresses just prior to Myf5) labels similar populations of cells with a few key differences. Notably, Pax3-Cre cells give rise to most of the brown adipocyte in iBAT, sBAT, cBAT, and prBAT, but none of the brown adipocytes in the paBAT (Sanchez-Gurmaches and Guertin 2014; Liu et al. 2013), and also to nearly 50% of the white adipocytes in the large visceral pgWAT depot. For comparison, MyoD-Cre (another classic myogenic transcription factor) does not label any brown or white adipocytes but importantly does label skeletal muscles (Sanchez-Gurmaches and Guertin 2014). Thus, there may be specificity within skeletal muscle lineages in which some precursors (i.e., Pax3/Myf5/Pax7^{positive}) can also become adipocytes while others (i.e., MyoD^{positive}) cannot or rather that some adipocyte and muscle precursors can independently express Pax3/Myf5/Pax7-Cre (see discussion above on the challenges of lineage-tracing studies) (Sanchez-Gurmaches and Guertin 2014; Haldar et al. 2008; Gensch et al. 2008). The most interesting possibility is that there is a temporal or spatial separation between certain lineages, and understanding this may help in understanding the commitment phase to brown adipocytes. Regardless, these studies conclusively revealed an unanticipated heterogeneity in both brown and white adipocyte development that suggests brown adipocytes residing in different depots could have different embryonic origins.

The developmental heterogeneity observed between brown adipocyte lineages is not likely due to low efficiency or specificity of the Cre drivers because independent experiments with Myf5-Cre, Pax3-Cre, and Pax7-CreER lines are remarkably similar (Lepper and Fan 2010; Sanchez-Gurmaches and Guertin 2014; Liu et al. 2013; Sanchez-Gurmaches et al. 2012; Seale et al. 2008; Shan et al. 2013; Wang et al. 2014b). Moreover, heterogeneous Myf5 labeling is also observed in skeletal muscle lineages in which Myf5 only labels around 50% of the satellite cells in the limb muscles but around 80% in epaxial muscles (Haldar et al. 2008; Gensch et al. 2008). An unanswered question is whether developmentally distinct brown (or white) adipocytes differ only in their anatomical location or whether they have unique functions (e.g., metabolic efficiency, BATokine production, exosome secretion, etc.) that might be specific by their developmental origins. Answering these questions will require an improved ability to isolate and study single brown

adipocytes, a deeper understanding of the regulatory mechanisms of BAT development, and markers that label the unidentified (Myf5-Cre; Pax3-Cre^{negative}) brown adipocyte lineages.

2.3 Postnatal and Adult Brown Fat Growth and Metabolism

In older laboratory mice (i.e., juveniles and adults), individual BAT depots can expand their mass by either increasing brown adipocyte number (hyperplasia) or by increasing individual cell size (hypertrophy) depending upon their initial housing temperature and the duration and degree of cold exposure. For example, hypertrophic growth of brown adipocytes is observed when mice living in standard housing conditions (22°C) are acclimated to their thermoneutral zone (e.g., 30–32°C). Under these conditions, the sympathetic tone is reduced by removing thermal stress, and the brown adipocytes decrease their thermogenic activity. This results in lipids accumulating and coalescing into a single large unilocular lipid droplet, thereby increasing individual cell size. Notably, while thermoneutral BAT displays a WAT-like morphology and gene expression signature, it maintains its BAT epigenetic signature (Hung et al. 2014; Veniant et al. 2015; Roh et al. 2018). Nevertheless, the net result of increasing cell size is in an increase in total depot size compared to mice living in the mild cold temperatures of most mouse facilities (Fig. 2).

Conversely, if mice living at thermoneutrality are moved to the mild cold (20–22°C) and BAT thermogenesis is activated, the mobilization and metabolism of lipids and other metabolites reduce individual adipocyte cell size and thereby overall BAT depot size (Fig. 2). However, if these mice are then further adapted to more severely cold temperatures (e.g., in 4–6°C range), additional new active brown adipocytes are recruited into the BAT depots (presumably from the brown ASPC pool described above), which increases BAT mass but by hyperplastic growth (Bukowiecki et al. 1982; Rehnmark and Nedergaard 1989; Geloen et al. 1992; Lee et al. 2015; Razzoli et al. 2018). Indeed, de novo adipogenesis of brown adipocyte precursor cells occurs in response to chronic cold (Rosenwald et al. 2013; Lee et al. 2015). Brown adipocyte size also increases by denervation, during extended high caloric (fat) feeding, or with aging (Hung et al. 2014; Roberts-Toler et al. 2015). Thus, while there is an underlying natural turnover of brown adipocytes (Sakaguchi et al. 2017), the iBAT depots in laboratory mice are smallest when mice are acclimated to standard lab conditions (mild cold), and it grows with increased or decreased temperature mainly by hypertrophic or hyperplasic growth, respectively.

Gene expression profiling of BAT tissue reveals greater differences between mice acclimated to thermoneutrality (30–32°C) and mild cold (20–22°C) than between mice acclimated to mild cold (20–22°C) and severe cold (6°C) (Sanchez-Gurmaches et al. 2018). This is consistent with brown fat morphology at these temperatures, which shows individual brown adipocytes in an "off" state (unilocular) in thermoneutrality and an "on" state (multilocular lipid droplets) at 22°C. Further reductions in temperature (e.g., to 6°C) increase the magnitude of thermogenesis and many genes associated with thermogenesis, and this is additionally reflected by morphological "ordering" of the lipid droplets (Fig. 2). A survey of metabolic